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Technique for Enumeration of Heterotrophic and Phototrophic Nanoplankton, Using Epifluorescence Microscopy, and Comparison with Other Procedures†

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A new method is described that uses the fluorochrome primulin and epifluorescence microscopy for the enumeration of heterotrophic and phototrophic nanoplankton (2 to 20 μm). Phototrophic microorganisms are distinguished from heterotrophs by the red autofluorescence of chlorophyll *a*. Separate filter sets are used which allow visualization of the primulin-stained nanoplankton without masking chlorophyll *a* fluorescence, thus allowing easy recognition of phototrophic cells. Comparison with existing epifluorescence techniques for counting heterotrophic and phototrophic nanoplankton shows that primulin provides more accurate counts of these populations than the fluorescein isothiocyanate or proflavine techniques. Accuracy is comparable to that with the acridine orange technique, but this method requires only one filter preparation for the enumeration of both phototrophic and heterotrophic populations.

Heterotrophic microflagellates (2 to 20 μm) are a ubiquitous component of plankton communities (6–8, 10, 17, 25, 28, 35–37, 40). Several studies have demonstrated the importance of these protozoa as predators for heterotrophic bacteria, chroococcoid cyanobacteria, and phototrophic nanoplankton (12–15, 20, 21, 23, 28, 32). Heterotrophic microflagellates play a role in the decomposition and remineralization of organic matter (12, 13, 29, 39) and are a potential food source for filter-feeding zooplankton (24). Although recent attempts have been made to incorporate these organisms into models of planktonic food webs (26, 30, 33, 36, 42), most investigations of the plankton still make no effort to determine the number of these protozoa present in nanoplankton samples (2, 3); hence, all microflagellates are considered phototrophic or osmotrophic.

Major problems with counting heterotrophic microflagellates in natural samples have been the accurate enumeration of the total nanoplankton and the differentiation of the organisms which lack photosynthetic pigments. A variety of counting techniques have been used for the nanoplankton, including the classical Utermöhl settling technique (41), microscopic observation of live samples (35, 37, 40), scanning electron microscopy (4, 34), and, more recently epifluorescence microscopy (11, 20, 28).

The first three counting techniques listed above have serious limitations with heterotrophic microflagellates. The Utermöhl technique underestimates nanoplankton concentrations by more than an order of magnitude (4, 11), presumably due to slow settling rates of small cells. Microscopic examination of live unconcentrated samples may be applicable in some situations, but it has been shown to underestimate the number of small flagellates due to restrictions on the magnification (4). The need to process samples immediately and to have high flagellate concentrations (to insure accurate and reproducible counts) renders this technique impractical for routine use. Scanning electron microscopy has been used successfully for studying nanoplankton samples, but it is time consuming and costly, and is impossible at sea.

A more important weakness of these techniques is their poor capability (or inability) for differentiating pigmented from nonpigmented nanoplankton. The taxonomic diversity of the heterotrophic nanoplankton has complicated this problem. Many phytoplankton taxa contain nonpigmented species (31), and therefore, cell morphology cannot be used as a tool to distinguish pigmented and nonpigmented organisms.

A more obvious method for distinguishing phototrophic from heterotrophic organisms is the presence or absence of photosynthetic pigments, a characteristic readily determined by epifluorescence microscopy. Several techniques involving epifluorescence microscopy have recently been proposed (11, 20, 28). These techniques rely on secondary fluorescence of vari-

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ous fluorochrome stains (acridine orange [AO], 3-6-diaminoacridine hemisulfate [proflavine], and fluorescein isothiocyanate [FITC], respectively) to make the heterotrophic nanoplankton visible and on the red autofluorescence of chlorophyll *a* to distinguish phototrophic nanoplankton. Ideally, it should be possible to distinguish fluorochrome from chlorophyll *a* fluorescence and to enumerate both groups of organisms from the same preparation. However, overlapping or interference of the emission spectra of the fluorochrome and chlorophyll *a* may result in masking of the chlorophyll fluorescence, resulting in error in the estimation of the proportion of phototrophic to heterotrophic organisms. This problem is most apparent in the AO technique (11), in which fluorochrome and chlorophyll *a* emission spectra overlap considerably. This necessitates two distinct counts on separate preparations: an unstained sample for chlorophyll *a* fluorescence only (phototrophic nanoplankton), and a stained sample for total nanoplankton (phototrophs and heterotrophs). The number of heterotrophic organisms is then determined by the difference (11). The proflavine and FITC techniques have been proposed to permit both counts on a single preparation. However, the fluorescence of these fluorochromes must be distinguished from that of chlorophyll *a* by eye, and there can be considerable masking of chlorophyll *a* fluorescence by these fluorochromes (11).

This paper describes an alternative staining procedure for simultaneously counting phototrophic and heterotrophic nanoplankton by epifluorescence microscopy. This procedure uses the fluorochrome primulin, which has excitation and emission maxima that are much lower than the corresponding maxima for chlorophyll *a*, allowing different filter combinations to be used to preferentially stimulate and observe either fluorochrome or chlorophyll *a* fluorescence. This method minimizes masking of chlorophyll *a* fluorescence and the resultant error in counting heterotrophic and phototrophic nanoplankton from a single preparation.

MATERIALS AND METHODS

All seawater samples were preserved with prefiltered (0.22-μm Millipore filter) 10% glutaraldehyde prepared in natural seawater to obtain a final preservative concentration of 1%. Freshwater samples were preserved at the same final concentration by using 10% glutaraldehyde prepared in distilled water with 0.1 M sodium cacodylate buffer (pH 7.0).

The fluorochrome primulin (Direct Yellow 59, Color Index 49000, Aldrich Chemical Co.) was prepared at a concentration of 250 μg/ml in distilled water with 0.1 M Trizma-hydrochloride at pH 4.0, since binding and fluorescence of the fluorochrome occur optimally at acid pH (9). The solution was prepared fresh and was filtered through a 0.22-μm Millipore filter before each use. Each preserved sample (10 to 50 ml) was drawn down onto a 0.8-μm, 25-mm Nuclepore filter prestained with irgalan black (22) at a vacuum not greater than 10 cmHg (13,330 Pa). A 0.5-μm, 25-mm Millipore filter was placed underneath the Nuclepore filter to promote even dispersion of the sample on the Nuclepore filter (27). The filter was then rinsed with two 1-ml portions of a rinse solution (distilled water with 0.1 M Trizma-hydrochloride [pH 4.0] filtered through a 0.22-μm Millipore filter), flooded with the primulin solution, and stained for 15 min without vacuum. The stain was then removed by gentle vacuum, and the filter was rinsed with two 2-ml portions of the rinse solution. The filter was removed and placed on a thin film of immersion oil (Cargille type A) on a glass slide, specimen side up. One drop of oil was placed on the center of the filter, followed by a cover slip.

A Zeiss standard microscope equipped with an HBO-50 mercury burner was used for all observations. A Neofluar ×100 objective lens (Planachromat and Planapochromat lenses will not work) and ×10 eyepieces were employed. A ×40 objective was found to be insufficient to determine detail on small (less than 5 μm) nanoplankton. Filter sets used for the observation of fluorescence were as follows: for primulin, a G365 exciter filter, an FT420 chromatic beam splitter, and an LP418 barrier filter (Zeiss filter set 487702); and for chlorophyll *a*, a BP450-490 exciter filter, an FT510 chromatic beam splitter, and an LP520 barrier filter (Zeiss filter set 487709). These filter sets were mounted in a filter insert housing (Zeiss 466301), which allowed their rapid exchange.

Two methods of enumeration were used. For estuarine and nearshore samples and laboratory cultures, microorganisms per field of view were counted. Fields were viewed first for primulin fluorescence to locate nanoplankton cells, and then for chlorophyll *a* fluorescence (by changing the filter set) to determine which of these cells were pigmented. The average number of cells per field of view was converted to organisms per milliliter by knowing the sample volume (×0.9, due to dilution during preservation), the area of the field of view, and the area of the filter covered by sample, using the equation: number of cells per ml = [(number of cells/field)(funnel area/field area)]/[(0.9)(sample volume)]. For oceanic samples, repeated scans across the filter were made at ×1,000. The length of each scan (in millimeters) was determined by using the mechanical stage divisions, and the area observed was determined by the scan length multiplied by the field diameter. Repeated interchange of the filter sets during each scan allowed determination of pigmented and nonpigmented cells. Care must be taken when scans are made using the ×100 Zeiss Neofluar objective. This lens does not produce a completely flat field, and constant focusing is necessary to see cells near the edge of the field. The error generated by these two counting methods was comparable; the averaged coefficient of variation for both methods together was 10.8% for 80 population counts.

I compared the accuracy of the primulin staining procedure with hemacytometer counts of five clonal cultures of phototrophic microflagellates and eight clonal cultures of heterotrophic microflagellates. The species of algae used were *Isochrysis galbana* (strain ISO), *Dunaliella tertiolecta* (DUN), *Monochrysis luth-*

eri (MONO), *Chroomonas salina* (3C), and *Micromonas* sp. (DW-8) from the culture collection of R. R. L. Guillard. Five of the heterotrophic microflagellates were marine: two species were isolated from coastal waters off Woods Hole, Mass. (*Paraphysomonas* sp. and *Bicoeca* sp.), and three species were isolated from the Sargasso Sea (*Rhynchomonas* sp. and two species of *Bodo*). The three remaining heterotrophs (*Actinomonas* sp. and two species of *Bodo*) were freshwater species isolated from Lake Ontario. All heterotrophs were cultured on a mixed bacterial flora enriched by adding 0.01% yeast extract to natural seawater or natural lake water. Phytoplankton were cultured in enriched seawater medium f/2 (19). Phytoplankton and protozoa were sampled during exponential growth and preserved in glutaraldehyde. Counts were made by using a Hausser Hy-Lite hemacytometer and with the primulin epifluorescence technique. Most samples were diluted for the fluorescence technique.

I also compared the existing procedures for epifluorescence microscopy used to enumerate phototrophic and heterotrophic nanoplankton. Natural samples from coastal and oceanic environments were counted by the AO technique (11), the FITC technique (28), the proflavine technique (20), and the primulin technique. There were minor changes in the other methods. (i) The Zeiss standard microscope equipped with an HBO-50 mercury burner was used instead of a 100-W tungsten-halogen lamp (20) or an Olympus Vanox microscope (11). (ii) Proflavine was added before preservation for only three samples (20), and Cargille type A immersion oil was substituted for type LF (20), since use of the latter had no effect on the counts.

The emission spectra of acridine orange, proflavine, FITC, and primulin were examined by using a Perkin-Elmer MPF-3 fluorescence spectrophotometer. Emission spectra between 520 and 700 nm were recorded for all four fluorochromes irradiated at 470 nm with a slit width of 40 nm. An emission slit width of 15 nm was used. The excitation wavelength and slit width were chosen to simulate the BP450-490 filter. The range of emission wavelengths represents visible light passing the barrier filter (LP520). The emission spectrum for primulin between 420 and 700 nm was examined at an excitation wavelength of 365 nm (slit width, 40 nm). This excitation wavelength and slit width simulate the G365 filter, and the emission spectrum represents visible light passing the barrier filter (LP418).

RESULTS AND DISCUSSION

Determination of the number of phototrophic nanoplankton is vital to the accurate enumeration of heterotrophic nanoplankton. If chlorophyll *a* fluorescence cannot be observed in an organism, it is counted as a heterotroph, and an incorrect assessment of the proportion of heterotrophic and phototrophic organisms results. Examination of unstained preparations for primary fluorescence of chlorophyll *a* remains the most effective method to determine the presence or absence of chlorophyll *a*, since no fluorochrome is present to obscure chlorophyll *a* fluorescence (6, 7, 11). However, potential problems with this technique include counting paired or multiple chloroplasts within a single organism as more than one organism, and underestimation of cell size (since the surrounding cytoplasm is often nonfluorescing). Furthermore, obtaining accurate counts of heterotrophs by determining the difference between total nanoplankton and phototrophic nanoplankton (AO technique; 11) is difficult in environments in which the phototrophs greatly outnumber the heterotrophs. Slide-to-slide variability may also affect the ratio of phototrophs to heterotrophs, since the AO procedure requires the preparation and enumeration of two filters per sample.

The proflavine and FITC procedures were developed to allow simultaneous counting of phototrophic and heterotrophic nanoplankton by using a fluorochrome stain which does not mask the red chlorophyll *a* fluorescence. These techniques have simplified counting of heterotrophic nanoplankton, but the emission spectra for these fluorochromes indicate a potential for masking of chlorophyll *a* fluorescence.

Figure 1 shows the emission spectra of AO, FITC, proflavine, and primulin under blue light excitation (Fig. 1B) and the emission spectrum for primulin under UV excitation (Fig. 1A). The unsuitability of AO as a means of identifying photosynthetic nanoplankton is shown by its relatively long wavelength emission spectrum. In theory, proflavine and FITC should not mask chlorophyll *a*, since their emission spectra (Fig. 1B) are similar and quite distinct from the peak of chlorophyll *a* fluorescence at 668 nm (18). However, the use of a single filter set with these fluorochromes means that the light from chlorophyll *a* fluorescence, albeit a different color, must be visible within the brightly fluorescing cytoplasm.

Primulin fluoresced brightly, with a maximum at ca. 425 nm, when excited with UV light (Fig. 1A). Primulin-stained cells fluoresced a bluish-white color under UV excitation. Flagella were visible on many cells in the seawater samples, although they were often not immediately apparent due to orientation or depth of field at 1,000×. The fluorescence of primulin under blue light excitation (BP450-490, used for chlorophyll *a* excitation) is substantially reduced (Fig. 1B) relative to fluorescence under UV excitation (Fig. 1A), making chlorophyll *a* fluorescence more obvious in primulin-stained cells. Thus, an examination for chlorophyll *a* fluorescence can be made on the same specimen simply by changing the filter set, due to the relatively nonoverlapping excitation and emission spectra of primulin and chlorophyll. The efficacy of this procedure relies on a means of easy exchange of the two filter sets.

Shifts in the emission spectra observed in Fig.

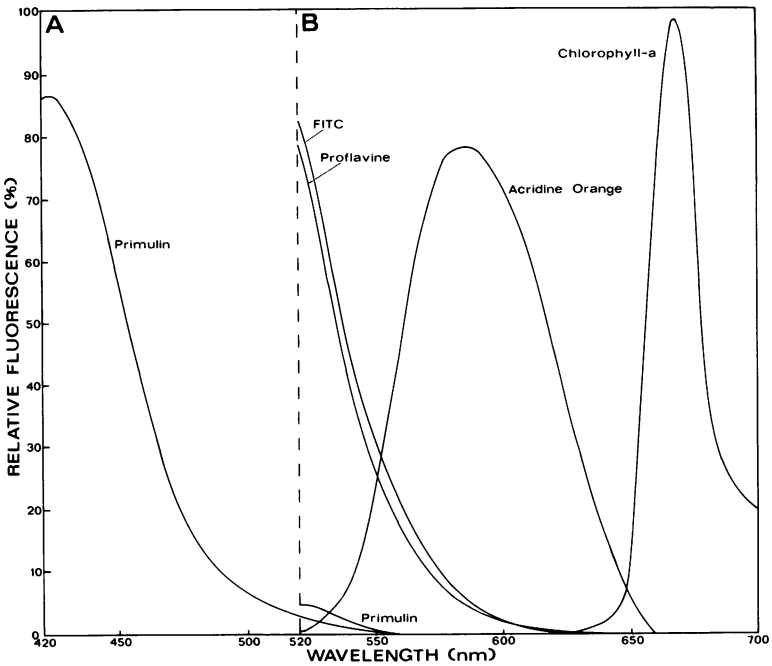

FIG. 1. Fluorescence emission spectra of primulin, proflavine, FITC, AO, and chlorophyll *a*. (A) Emission spectrum of primulin when excited at 365 nm with a slit width of 40 nm. (B) Emission spectra of primulin, proflavine, FITC, and AO when excited at 470 nm with a slit width of 40 nm. The same concentration of primulin was used for both types of excitation. The chlorophyll *a* emission spectrum was taken from French et al. (18).

1 may occur due to binding of the fluorochrome or changes in the exact spectral quality of the excitation beam. The extent of these differences is unknown. However, Haas (20) noted that proflavine caused detritus to fluoresce pink. This suggests a shift to longer emission wavelengths.

Staining characteristics of primulin, AO, FITC, and proflavine are shown for a small phytoflagellate (*Micromonas* sp.) in Fig. 2. Unstained preparations (Fig. 2A) show the red autofluorescence of chlorophyll *a*. Organisms stained with AO (Fig. 2B) fluoresce bright orange to red, masking chlorophyll *a* fluorescence. Primulin-stained preparations (Fig. 2C) excited the UV light fluoresce brightly, but under blue light (Fig. 2D) fluorescence is greatly reduced, making the chlorophyll *a* easily visible. Chlorophyll *a* fluorescence of FITC-stained (Fig. 2E) and proflavine-stained (Fig. 2F) preparations was less apparent than for primulin-stained preparations. Although the colors of the FITC (green) and proflavine (yellow) are distinct from the red chlorophyll *a* fluorescence, the intensity of the fluorochrome fluorescence masks that of chlorophyll *a*, especially in small cells (Fig. 2). Chlorophyll *a* fluorescence in larger phototrophic nanoplankton is more intense, and not as easily masked by the fluorochrome.

A comparison between the counts obtained by hemacytometer and by primulin epifluorescence is given in Table 1. The counts were not significantly different (Wilcoxon rank sum test, $\alpha = 0.05$) for the microalgae and protozoa tested.

A comparison of the AO, FITC, proflavine, and primulin counts of 10 nearshore and oceanic samples is given in Table 2. Unstained preparations (no fluorochromes added) were counted for the number of phototrophic nanoplankton as part of the AO technique and were used as a means of comparing the counts of phototrophic nanoplankton for the FITC, proflavine, and primulin methods. The AO counts were assumed to indicate total nanoplankton (11) to which the other techniques were compared.

The results of the primulin technique agreed well with those of the AO technique. In one sample, the count of phototrophic cells was significantly greater than that in the unstained preparation, and in one sample, the total nanoplankton were significantly fewer than with the AO count. In contrast, the FITC counts indicated significantly fewer phototrophic cells than the unstained counts in nine samples. Total nanoplankton for this staining procedure were also significantly fewer than the AO counts in nine samples, indicating an underestimation of both phototrophic and total nanoplankton by the FITC technique. Averages for the 10 samples by the FITC technique were 56% of the number of

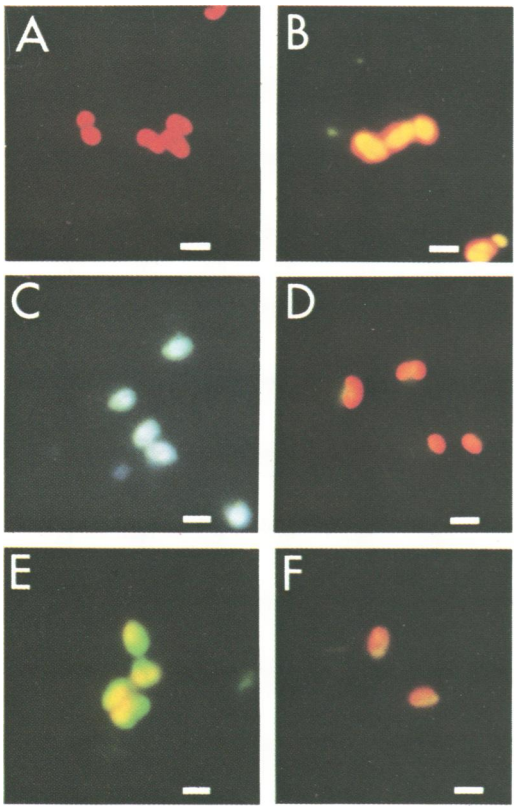

FIG. 2. Photomicrographs of *Micromonas* sp. (A) Unstained preparation showing autofluorescence of chlorophyll. (B) AO-stained preparation. Note that the orange color of the AO masks the red chlorophyll fluorescence. (C and D) Primulin-stained preparation irradiated with UV light (C) to preferentially excite primulin and blue light (D) to preferentially excite chlorophyll *a*. (E) FITC-stained preparation. (F) Proflavine-stained preparation. Bar, 2 μm.

phototrophic nanoplankton (relative to unstained preparations) and 64% of the number of total nanoplankton (relative to the AO preparations). FITC stains some detritus intensely. Many organisms in the samples were associated with particles, and it is probable that they were not visible because of this high background fluorescence. This might explain why fewer phototrophic nanoplankton (which should have been visible due to chlorophyll *a* autofluorescence) were observed in nine samples relative to the unstained preparations. Sherr and Sherr (28) noted a decrease in the number of heterotrophic and phototrophic nanoplankton (as shown by the FITC technique) in preserved samples of 46 and 18%, respectively, after 4 weeks of storage at 5°C, but they observed no decrease after 2 weeks of storage. Alternatively, the results for the FITC technique (Table 2) may indicate that these decreases occur sooner than 2 weeks for natural samples.

Four proflavine-stained samples had significantly fewer phototrophic cells relative to unstained preparations, whereas only two samples had significantly fewer total nanoplankton than AO counts (Table 2). This suggests that some phototrophic nanoplankton were included in the counts of heterotrophs. Staining of detritus may also explain some of the discrepancies in the proflavine counts. Proflavine often causes detritus to fluoresce pink (20), leading to an inability to distinguish chlorophyll *a* fluorescence in phototrophic organisms on or near particles. In one sample, the number of phototrophic cells exceeded that in the unstained preparation. This was the same sample that gave a higher phototroph count with the primulin method.

The inevitable result of overstaining with proflavine and FITC is a masking of chlorophyll *a* fluorescence, since the same filter set is used for fluorochrome and chlorophyll *a* excitation. Overstaining can cause the incorrect identification of phototrophs as heterotrophs, resulting in an underestimation of the number of phototrophs and an overestimation of the number of heterotrophs. Understaining can result in an underestimation of the number of heterotrophs and, in turn, in the total number of nanoplankton, since poorly stained cells may be overlooked. Both of these artifacts (underestimation of phototrophs and underestimation of total nanoplankton) are apparent in Table 2 for the proflavine and FITC procedures. Primulin allows slight overstaining of nanoplankton without significant masking of chlorophyll fluorescence due to the minimal overlap between primulin

TABLE 1. Comparison of direct counts of clonal cultures for five species of photosynthetic flagellates and eight species of heterotrophic flagellates

Species	Direct count ($\times 10^3$ cells per ml) as measured by:	
	Hemacytometer	Primulin
Phototrophs		
Isochrysis galbana	4.09	4.03
Dunaliella tertiolecta	6.12	6.19
Monochrysis lutheri	3.74	3.81
Chroomonas salina	7.70	7.89
Micromonas sp.	82.8	86.9
Heterotrophs		
Paraphysomonas sp.	2.72	2.44
Bicoeca sp.	2.15	2.22
Rhyncomonas sp.	2.91	2.67
Bodo sp. 1	1.08	1.05
Bodo sp. 2	10.2	10.1
Actinomonas sp.	1.87	1.80
Bodo sp. 1 (freshwater)	4.91	4.97
Bodo sp. 2 (freshwater)	0.24	0.20

TABLE 2. Counts of photosynthetic (Pnano), heterotrophic (Hnano), and total (Tnano) nanoplankton for four nearshore and six oceanic stations[a]

Sample description (date and location)	Parameter	Nanoplankton count in the following prepn:				
		Unstained	AO	Primulin	FITC	Proflavine
Eel Pond	Pnano	4.12×10^3		4.24×10^3	1.86×10^{3}*	2.70×10^{3}*
10/8/82	Hnano			1.51×10^3	1.98×10^3	1.65×10^3
Woods Hole, Mass.	Tnano		5.45×10^3	5.75×10^3	3.84×10^{3}*	4.36×10^3
Vineyard Sound	Pnano	5.08×10^3		5.13×10^3	3.40×10^{3}*	4.35×10^{3}*
10/13/82	Hnano			1.71×10^3	1.70×10^3	1.48×10^3
Falmouth, Mass.	Tnano		6.86×10^3	6.84×10^3	5.10×10^{3}*	5.83×10^{3}*
Head of Buzzards Bay	Pnano	1.79×10^3		1.94×10^3	1.46×10^{3}*	1.77×10^3
10/12/82	Hnano			2.34×10^3	1.06×10^3	2.34×10^3
Buzzards Bay, Mass.	Tnano		4.19×10^3	4.28×10^3	2.52×10^{3}*	4.12×10^3
Woods Hole Harbor	Pnano	3.04×10^3		3.24×10^3	2.61×10^3	2.47×10^3
10/11/82	Hnano			2.14×10^3	2.68×10^3	2.31×10^3
Woods Hole, Mass.	Tnano		5.50×10^3	5.38×10^3	5.29×10^3	4.78×10^{3}*
Sargasso Sea (surface)	Pnano	2.04×10^2		2.58×10^2	5.24×10^{1}*	1.79×10^2
10/29/82	Hnano			7.03×10^2	2.56×10^2	6.38×10^2
34°44′ N 70°00′ W	Tnano		8.06×10^2	9.61×10^2	3.09×10^{2}*	8.17×10^2
Sargasso Sea (surface)	Pnano	2.43×10^2		3.66×10^2	1.60×10^{2}*	2.17×10^2
10/30/82	Hnano			1.24×10^3	6.79×10^2	1.17×10^3
33°57′ N 70°00′ W	Tnano		1.38×10^3	1.61×10^3	8.40×10^{2}*	1.38×10^3
Sargasso Sea (surface)	Pnano	2.26×10^2		2.34×10^2	9.18×10^{1}*	1.44×10^{2}*
10/31/82	Hnano			2.42×10^2	4.20×10^2	5.46×10^2
34°04′ N 70°08′ W	Tnano		7.52×10^2	4.76×10^{2}*	5.11×10^{2}*	6.90×10^2
Sargasso Sea (surface)	Pnano	2.27×10^2		3.33×10^{2}+	1.29×10^{2}*	3.51×10^{2}+
11/2/82	Hnano			6.63×10^2	4.60×10^2	9.00×10^2
35°40′ N 69°33′ W	Tnano		1.19×10^3	9.96×10^2	5.88×10^{2}*	1.25×10^3
Sargasso Sea (20 meters)	Pnano	5.10×10^2		4.87×10^2	3.56×10^{2}*	5.24×10^2
11/2/82	Hnano			4.50×10^2	4.30×10^2	3.76×10^2
36°26′ N 69°14′ W	Tnano		9.88×10^2	9.37×10^2	7.86×10^{2}*	9.00×10^2
Continental shelf edge (surface)	Pnano	5.44×10^3		4.67×10^3	1.17×10^{3}*	3.09×10^{3}*
11/4/82	Hnano			1.68×10^3	1.67×10^3	2.87×10^3
39°43′ N 70°13′ W	Tnano		6.43×10^3	6.35×10^3	2.84×10^{3}*	5.96×10^3

[a] Counts of unstained and AO-stained preparations were used as an index of Pnano and Tnano for comparison of the FITC, proflavine and primulin techniques. An asterisk (*) indicates a significantly lower count (Wilcoxon rank sum test, $\alpha = 0.05$) relative to the unstained or AO count. A plus (+) indicates a significantly higher count than the unstained or AO count. The Eel Pond, Head of Buzzards Bay, and Woods Hole Harbor samples were stained with proflavine before preservation (20). The remaining samples were stained after preservation.

and chlorophyll excitation and emission spectra.

Masking of chlorophyll *a* fluorescence may be augmented by the gradual loss of the intensity of chlorophyll *a* fluorescence in preserved samples, and by the predominance of small (less than 5 μm) phototrophic organisms. The latter situation was noted by Davis and Sieburth (11), who observed fewer phototrophic nanoplankton when using proflavine (relative to the unstained/AO technique). This difference was particularly acute for cells 2 to 3 μm in diameter. The Sargasso Sea samples enumerated in this study were 4 to 8 days old when counted, and both age and small cell size may have caused some of the observed discrepancies among techniques (Table 2).

The primulin technique proposed here provides a more accurate method (although slightly more complicated) than the FITC and proflavine techniques for the enumeration of phototrophic and heterotrophic nanoplankton. The use of two filter sets which can be easily and quickly exchanged is a necessity for the primulin method. This is not a requirement for the proflavine and FITC methods. Proflavine may also be used to count bacteria present in a sample simultaneous-

ly (20), although these counts appear to be inferior to DAPI counts (P. W. Johnson and J. M. Sieburth, personal communication). Although primulin has been used to identify algae (5), its accuracy for bacterial enumeration is doubtful (unpublished data). The AO method provides accuracy comparable to that of the primulin method, but the need for two preparations per sample and other potential problems previously mentioned reduce its usefulness relative to the primulin method.

A recent goal of direct counting techniques for heterotrophic nanoplankton has been an accurate appraisal of the number of bacterivorous microflagellates in plankton communities (17, 28). As with most assemblages of organisms based on size classes, however, the heterotrophic nanoplankton is a heterogeneous assemblage. In addition to bacterivorous microflagellates and their cysts (recent work by Fenchel [16] suggests that cysts are of minor importance for nearshore communities), some microflagellates are also capable of ingesting phytoplankton nearly their own size (20). The heterotrophic nanoplankton may also include such diverse groups as the gametes of planktonic protozoa or metazoa (1, 38) and those of brown algae and phycomycetes (31). Another unknown is the percentage of chlorophyll-containing microflagellates which partially or mostly function as heterotrophs (14). At present, these complications warrant caution in the use of direct counts as an unequivocal measure of bacterivorous microflagellate densities and in the subsequent use of these numbers to estimate the impact which microprotozoa have on natural bacterial assemblages (17, 28).

Most-probable-number cultural methods generally yield estimates of bacterivorous microflagellates well below direct counts in oceanic waters (25; unpublished data). This is due in part to an inability to culture fastidious species of bacterivorous protozoa present in the open ocean. Fairly close agreement between most-probable-number estimates and direct counts has been observed for inshore waters (17). These latter results suggest that at least for inshore environments, the relative importance of bacterivorous microflagellates in the heterotrophic nanoplankton may be high. A more complete characterization of the trophic modes of microflagellate species in all plankton communities awaits investigation. At present, direct counting procedures represent the best available technique for the rapid and accurate estimation of this component of planktonic communities, and a means of investigating trophic relationships among planktonic microorganisms (6, 7).

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